DOI: 10.1002/cbic.200700643

Using Fluorous Amino Acids to Modulate the Biological Activity of an Antimicrobial Peptide

Lindsey M. Gottler,[a] Hyang-Yeol Lee,[a] Charles E. Shelburne,[b] Ayyalusamy Ramamoorthy,*[a] and E. Neil G. Marsh*[a]

The emergence of bacterial strains resistant to most of the clinically useful antibiotics has provided the impetus to develop new classes of antibiotics that might combat bacterial resistance more effectively. Antimicrobial peptides (AMPs) are small peptides (typically 15–30 residues) that show promise as therapeutic agents against bacteria, fungi, and viruses.[1–3] Widely distributed in plants and animals, they form part of the innate immune system's defense against microbes. Although highly diverse in sequence and structure, almost all AMPs share the property of being highly amphiphathic: one face of the peptide is hydrophobic and the other face presents a cluster of positively charged residues.[4,5] AMPs function by disrupting bacterial membranes,[4] which contain predominantly negatively charged phospholipids. Eukaryotic membranes, which contain predominantly neutral phospholipids, are not targeted.

Although promising as broad-spectrum antibiotics, AMPs are susceptible to proteolysis in vivo by endogenous or bacterial proteases, which can considerably diminish their effectiveness. Attempts to overcome this problem by increasing the dose of AMP often leads to toxic side effects, most notably lysis of red blood cells, which has been attributed to nonspecific hydrophobic interactions between the peptide and the eukaryotic cell membrane.[6,7] Here we describe a strategy to overcome these limitations, by exploiting the unusual physicochemical properties exhibited by fluorocarbons.

Fluorocarbons are noted for their chemical inertness and their extreme hydrophobicity. Fluorocarbon solvents exhibit unusual self-segregating properties, known as the fluorous effect, which has been exploited in organic synthesis to facilitate the extraction of organic molecules "tagged" with fluorocarbon chains from organic solvents.[8] Work in our laboratory and others has shown that extensively fluorinated analogues of leucine and valine can significantly stabilize small proteins against thermal and chemical denaturation,[9–15] an effect that can be attributed to the extremely hydrophobic nature of fluorocarbons. Although fluorinated amino acids have been incorporated into AMPs,[16] so far extensively fluorinated or fluorous amino acids have not been used to modify the biological properties AMPs. We reasoned that if these properties of fluorocarbons could be designed into AMPs, then the expected increased structural stability might provide resistance to proteolysis, without abolishing their biological activity or increasing their toxicity to eukaryotic cells.

To test this concept we have synthesized a fluorous analogue of the potent and well-characterized AMP, MSI-78 (also called pexiganan). MSI-78 is a synthetic analogue of magainin-2, an α-helical AMP originally isolated from *Xenopus laevis*, that has potent antibacterial activity.[17,18] The peptide is unstructured in free solution but forms a dimeric antiparallel α-helical coiled-coil on association with lipid bilayers[19] (Figure 1) and is

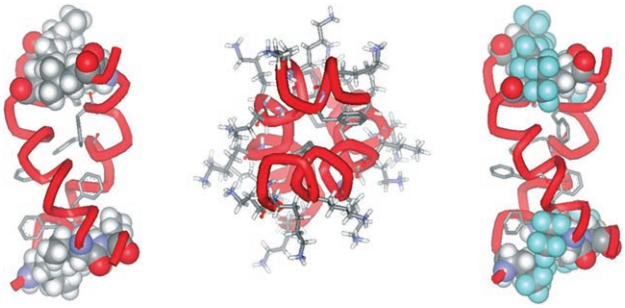

Figure 1. Left: Structure of MSI-78 dimer showing Leu and Ile residues in CPK rendering. Center: View along the helical axis of MSI-78 dimer showing disposition of Lys residues. Right: model of fluorogainin-1 based on structure of MSI-78 showing hFleu residues in CPK rendering.

believed to exert its antibacterial effect by forming toroidal pores in the bacterial membrane.[20] We have replaced the two leucine and isoleucine residues in MSI-78 with the fluorous amino acid L-5,5,5,5′,5′,5′-hexafluoroleucine (hFLeu) to produce a molecule we call fluorogainin-1 (Figure 1). The sequences of these peptides are shown below (where X = hFLeu):

MSI-78: GIGKFLKKAKKFGKAFVKILKK

fluorogainin-1: G**X**GKF**X**KKAKKFGKAFVK**XX**KK

We have compared the antimicrobial activity, hemolytic activity, and resistance to proteolysis of fluorogainin-1 and MSI-78. We have also examined the interactions of these peptides with lipids using isothermal titration calorimetry (ITC).

Fluorogainin-1 was synthesized manually by using Boc-protected amino acids by standard protocols;[21] Boc-protected hFLeu was synthesized as described previously.[22] As a control, MSI-78 was synthesized by standard automated methods as described previously.[23] Both peptides were purified by reversed-phase HPLC and their identities confirmed by MALDI-MS.

[a] L. M. Gottler, H.-Y. Lee, Prof. A. Ramamoorthy, Dr. E. N. G. Marsh
Department of Chemistry, University of Michigan
Ann Arbor, MI 48109 (USA)
Fax: (+1) 734-615-3790
E-mail: nmarsh@umich.edu
ramamoor@umich.edu

[b] C. E. Shelburne
Dental School, University of Michigan
Ann Arbor, MI 48109 (USA)

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We first compared the effect of fluorination on the antimicrobial activity of the two peptides. The minimum inhibitory concentration (MIC) of MSI-78 and fluorogainin-1 were determined by the microdilution antimicrobial assay procedure, as described previously.[24] A panel of eleven bacterial strains were chosen that included both Gram positive and Gram negative strains of common pathogenic bacteria. Twofold serial dilutions of each AMP were made into cultures of each bacterial strain and the minimum concentration needed to prevent bacterial growth was determined. The results are summarized in Table 1.

Table 1. MICs of MSI-78 and fluorogainin-1 against various bacterial strains.

Bacterial strain	MIC [$\mu g\,mL^{-1}$] MSI-78	fluorogainin-1
Bacillus subtilis	<4	8
Kocheria rhizophila	<4	8
Enterobacter aerogenes	>250	>250
Klebsiella pneumoniae	>250	16[a]
Proteus mirabilis	>250	>250
Salmonella enteritis	16	32
Streptococcus pyogenes	8	62[b]
Escherichia coli (DH5a)	<4	8
Staphylococcus aureus (UH-11)	62	16[a]
Shigella sonnei	16	32
Enterococcus fecaelis (OG1 X)	>250	>250

[a] MIC of fluorogainin-1 was significantly lower than that of MSI-78 ($p<0.05$); [b] MIC of fluorogainin-1 was significantly higher than that of MSI-78 ($p<0.05$).

The fluorous AMP retained the broad-spectrum antibiotic activity of MSI-78, even though the incorporation of eight trifluoromethyl groups into the peptide might be considered quite an extensive modification of the structure. Although MSI-78 appeared slightly more active than fluorogainin-1 against many of the bacteria, differences of twofold in MIC cannot be considered statistically significant in this type of dilution assay. However, fluorogainin-1 was significantly more potent ($p<0.05$) against two important pathogenic bacteria: the MIC of fluorogainin-1 against *Klebsiella pneumoniae* was 16 $\mu g\,mL^{-1}$ whereas MSI-78 showed no activity, and the MIC of fluorogainin-1 was approximately four-times lower than MSI-78 against *Staphylococcus aureus*. The only bacterium tested for which fluorogainin-1 was significantly ($p<0.05$) less effective than MSI-78 was *Streptococcus pyogenes*.

To determine whether fluorination might result in increased toxicity, the hemolytic activity of both AMPs was tested against sheep erythrocytes by using a standard lysis assay for hemoglobin release from erythrocytes as described previously.[25] Neither MSI-78 nor fluorogainin-1 exhibited any hemolytic activity at concentrations of up to 250 $\mu g\,mL^{-1}$. To place this result in context: many AMPs exhibit hemolytic activity at concentrations well below 100 $\mu g\,mL^{-1}$. It appears, therefore, that even though the hFLeu side chain is considerably more hydrophobic than Leu or Ile[12] this does not result in the peptide binding to erythrocyte membranes.

The secondary structure of the peptides was examined by CD spectroscopy. Peptide samples were prepared at 70 μM concentration in Tris-Cl buffer (100 mM), $CaCl_2$ (10 mM), pH 7.8, at 25 °C. In the absence of liposomes both peptides were unstructured (data not shown), which is consistent with their being highly positively charged. However, in the presence of small unilamellar vesicles (SUVs) prepared from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphotidylcholine (POPC, 14 mM final concentration) both peptides exhibited CD spectra characteristic of an α-helical structure (Figure 2). Interestingly, fluorogainin-1 appeared significantly less helical than MSI-78 as judged by the CD spectra, with a mean residue ellipticity at 222 nm of only about $^2/_3$ that of the nonfluorinated peptide. This is in accord with recent studies that have shown that hFLeu has a poor helix propensity,[26] despite the fact that it has been found to stabilize α-helical proteins against unfolding.

We next examined the stability of fluorogainin-1 and MSI-78 towards proteolysis by two common proteases, trypsin, and chymotrypsin. These proteases provide a stringent test for structural stability as both peptides have multiple potential cleavage sites for both enzymes. Peptides (350 μM) were dissolved in Tris-Cl buffer (100 mM), $CaCl_2$ (10 mM), pH 7.8, and were incubated in the presence of POPC (15 mM) liposomes and protease (1.25%, *w/w*) at 25 °C for various times and the extent of proteolysis was determined by reverse phase HPLC. Under these conditions MSI-78 was almost completely degraded by either protease within 30 min, however, fluorogainin-1 showed no signs of degradation even after 10 h (Figure 2). In the absence of liposomes, when the peptides are unstructured, both peptides were equally rapidly degraded by both trypsin and chymotrypsin. Therefore, it appears that the resistance of fluorogainin-1 to proteolysis is due to its interaction with liposomes and not because the incorporation of hFLeu per se prevents the peptide from being digested by proteases.

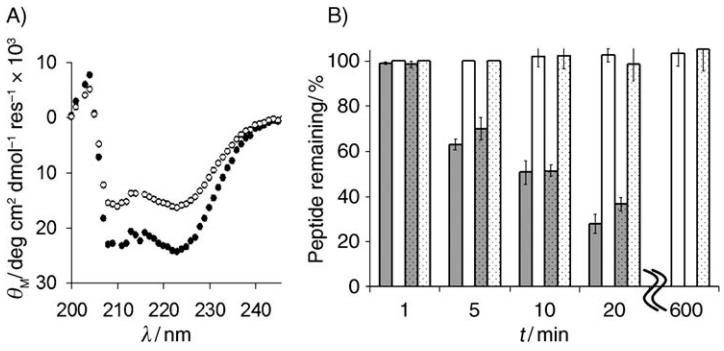

Figure 2. A) CD spectra for MSI-78 (●) and fluorogainin-1 (○) in the presence of POPC. B) Stability of MSI-87 (grey) and fluorogainin-1 (white) towards digestion with trypsin (solid bars) or chymotrypsin (dotted bars).

The interactions between liposomes and the AMPs were studied by isothermal titration calorimetry. Measurements were made under conditions similar to those used to study the

binding of the parent peptide magainin-2 to SUVs.[27] SUVs were freshly prepared from a 3:1 (*mol*/*mol*) mixture of POPC/POPG (final concentration 15 mM) in potassium phosphate buffer (12 mM, pH 7.4) containing NaCl (137 mM), thoroughly degassed, and introduced into the calorimeter cell. A solution of peptide (200 μM; dissolved in the same buffer) was injected in 4 μL increments into the liposome solution. Measurements were made at 25 °C by using a Microcal VP calorimeter. Heats of dilution were determined by injecting peptide solutions into buffer that lacked SUVs and subtracted from the raw data.

The thermograms for each peptide binding to liposomes are shown in Figure 3. The enthalpies (calculated from the peak areas of each injection of peptide) for each peptide bound to the lipids were similar: ΔH for MSI-78 was (-14.4 ± 0.2) kcal mol^{-1}, whereas ΔH for fluorogainin-1 was slightly less exothermic at (-12.5 ± 0.3) kcal mol^{-1}. These enthalpies are similar to those measured previously for magainin-2 peptide bound to SUVs.[27] The titration of fluorogainin-1 appears to have a slight increase in heat released with increasing injection number; this indicates some dependence on peptide concentration, although the significance of this is unclear. The enthalpic contribution to liposome binding has been determined to arise primarily from electrostatic interactions between the positively charged peptide and negatively charged lipid head groups.[27] Since MSI-78 and fluorogainin-1 contain identical cationic residues, the electrostatic interactions are expected to be very similar. This result strongly suggests that the increase in hydrophobicity imparted by the fluorous residues is primarily responsible for modifying the biological properties of fluorogainin-1.

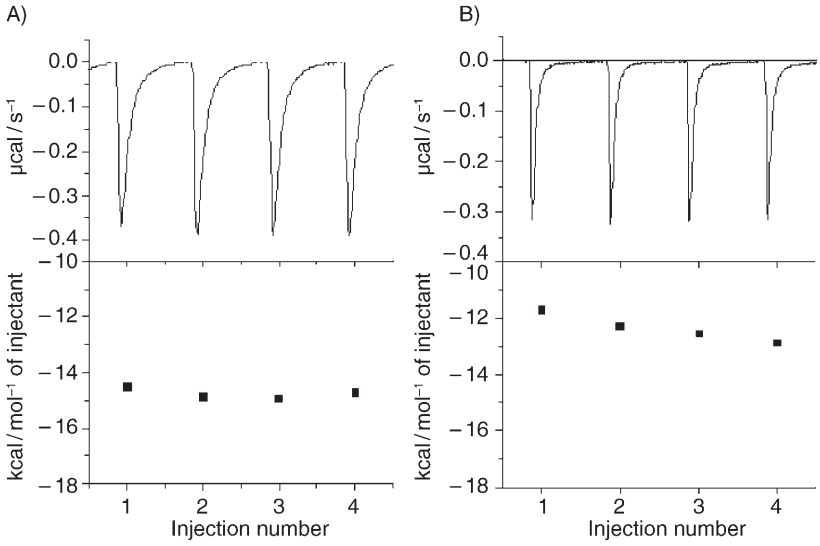

Figure 3. A) Isothermal titration calorimetry of MSI-78 into SUVs. B) Isothemal titration calorimetry of fluorogainin-1 into SUVs. In each case the upper panel shows heat of reaction for four consecutive 4 μL injections of peptide (200 μM) solution into a large molar excess of liposomes (1.2 mL, 15 mM); the lower panels show the integration of these peaks.

In conclusion, by introducing fluorous amino acid residues into an AMP we have conferred almost complete resistance to proteolysis of the fluorous AMP under conditions in which the nonfluorinated AMP is rapidly degraded, while retaining the broad spectrum of antimicrobial activity. Protection against proteolysis was only observed in the presence of liposomes; this suggests that lipid–peptide interactions are important. MSI-78 has been shown to dimerize to form a coiled-coil in a membrane environment.[19] Based on this observation, one plausible explanation of the protease resistance exhibited by fluorogainin-1 is that incorporation of the more hydrophobic hFLeu side chain strengthens the hydrophobic interactions between AMP dimers, just as we have demonstrated for other coiled-coil proteins.[12,13] This would, in turn, promote the formation of structured dimers that are resistant to proteolysis.

It is also noteworthy that fluorogainin-1 shows a selectivity towards bacterial strains that are slightly different from that of MSI-78. Fluorogainin-1 exhibited significantly improved potency against *K. pneumoniae* and *S. aureus*, with MICs of 16 μg mL^{-1} against both bacteria. Although the selectivity of AMPs for some bacteria and not others is poorly understood,[6] it is known that the resistance of *S. aureus* to AMPs is due, at least in part, to secretion of proteases.[28] The resistance of fluorogainin-1 to proteolysis might explain its improved potency against this important bacterial pathogen.

More generally, our results suggest the strategy of incorporating fluorous residues into biologically active membrane-associated peptides could be used to enhance the efficacy or modulate the activity of other biologically important peptides. For example, membrane-active peptides are known to be important in membrane fusion and ion-channel formation, and have also been found to have anticancer and antiviral activities.[29–32]

Acknowledgements

This work was supported in part by grants from the National Science Foundation (CHE 0640934) to E.N.G.M and the American Heart Association to A.R.

Keywords: antimicrobial agents · fluorine · isothermal titration calorimetry · membrane permeabilization · peptides

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Received: October 29, 2007
Published online on January 25, 2008

